What have we learned about GPER function in physiology and disease from knockout mice?

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Abstract

Estrogens, predominantly 17β-estradiol, exert diverse effects throughout the body in both normal and patho-physiology, during development and in reproductive, metabolic, endocrine, cardiovascular, nervous, musculoskeletal and immune systems. Estrogen and its receptors also play important roles in carcinogenesis and therapy, particularly for breast cancer. In addition to the classical nuclear estrogen receptors (ERα and ERβ) that traditionally mediate predominantly genomic signaling, the G protein-coupled estrogen receptor GPER has become recognized as a critical mediator of rapid signaling in response to estrogen. Mouse models, and in particular knockout (KO) mice, represent an important approach to understand the functions of receptors in normal physiology and disease. Whereas ERα KO mice display multiple significant defects in reproduction and mammary gland development, ERβ KO phenotypes are more limited, and GPER KO exhibit no reproductive deficits. However, the study of GPER KO mice over the last six years has revealed that GPER deficiency results in multiple physiological alterations including obesity, cardiovascular dysfunction, insulin resistance and glucose intolerance. In addition, the lack of estrogen-mediated effects in numerous tissues of GPER KO mice, studied in vivo or ex vivo, including those of the cardiovascular, endocrine, nervous and immune systems, reveals GPER as a genuine mediator of estrogen action. Importantly, GPER KO mice have also revealed roles for GPER in breast carcinogenesis and metastasis. In combination with the supporting effects of GPER-selective ligands and GPER knockdown approaches, GPER KO mice demonstrate the therapeutic potential of targeting GPER activity in diseases as diverse as obesity, diabetes, multiple sclerosis, hypertension, atherosclerosis, myocardial infarction, stroke and cancer.
Introduction

The physiology of estrogen and its multiple receptors is diverse and highly complex [1–4]. Estrogen is recognized predominantly for its function in the female reproductive system including the development of secondary sex characteristics, with actions predominantly in the uterus and mammary glands [5]. However, estrogen also plays multiple and critical roles in most physiological systems of the body [6], and importantly not only in women but also in men [7, 8]. Functions for estrogen in metabolism [9, 10], endocrine [11], cardiovascular [12], nervous [13], musculoskeletal [14] and the immune [15] system are all well documented. The actions of estrogen were traditionally thought to be mediated by a single estrogen receptor, now named estrogen receptor α (ERα, ESR1), first identified in the 1960’s [16, 17]. Not until 1996 was a second structurally homologous estrogen receptor identified [18], now named estrogen receptor β (ERβ, ESR2). These receptors are both members of the family of nuclear hormone receptors and are generally described to function primarily as ligand-regulated transcription factors [6, 19, 20]. However, as the earliest described cellular signaling activities of estrogen included cAMP production [21] and calcium uptake [22], it is clear that estrogen mediates rapid cellular signaling events as well as acting as a transcriptional regulator [23]. The status quo regarding estrogen receptor biology was upended in the 2000’s with the discovery and characterization of a third estrogen receptor, structurally unrelated to ERα/β [24–26]. This receptor, originally named GPR30, based on its orphan designation, and now named G protein-coupled estrogen receptor (GPER) [3], belongs to the family of 7-transmembrane G protein-coupled receptors (GPCRs), which classically mediates rapid cellular responses involving kinases, ion channels and second messengers [27].

Studies of these three estrogen receptors have enhanced our understanding of the extensive and complex activities of estrogen throughout the body [2, 4, 28, 29]. However, given that all three estrogen receptors mediate both rapid signaling and transcriptional regulation in response to estrogen [3, 30, 31], there remain many questions regarding the functions of each individual receptor, as well as their interactions, in the complex physiology of estrogen. One approach to address the functions of individual receptors has been through the development of receptor-selective pharmacological agents. Many compounds have been identified that exhibit selective activity within the classical estrogen receptors, either with respect to ER function in different tissues or between ERα and ERβ. An example of the former is the selective estrogen receptor modulator (SERM), which includes molecules such as tamoxifen and raloxifene, that act as antagonists in the breast but agonists in bone, based on the fundamental actions of estrogen in each of these tissues [32, 33]. ER antagonists that result in ER degradation in all tissues are referred to as selective estrogen receptor downregulators (SERDs) [32, 33]. Both types of agents have found widespread use in the treatment of breast cancer (SERMs and SERDs) as well as osteoporosis (SERMs), although they exhibit virtually no selectivity between ERα and ERβ. Compounds that do exhibit such
selectivity include 4,4′,4″-(4-propyl-1H-pyrazole-1,3,5-triyl) trisphenol (PPT), which exhibits ~400-fold selectivity for ERβ over ERα [34], and 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), which exhibits ~70-fold selectivity for ERβ over ERα [35]. Studies of GPER however have revealed that both SERMs [25, 36] and SERDs [24], as well as PPT [36] function as GPER agonists, thus complicating our understanding of estrogen action based on the use of such compounds [3]. In contrast, both a GPER-selective agonist (G-1, [37]) and antagonists (G15 and G36, [38, 39]) have been described that exhibit virtually no activity towards the classical estrogen receptors [3] and over 1000-fold selectivity towards a panel of 25 GPCRs [40], facilitating pharmacological studies of GPER function in cells, tissues and animals [2–4]. Although no “off-target” effects have been reported for G15 or G36, G-1 has been suggested to interact with microtubules at high concentrations [41–43]. It is important to remember however that every small molecule pharmacologic agent will likely exhibit “off-target” effects, particularly at “high” concentrations (≫EC50 of the intended target) [44]. As in the words of Paracelsus: “The dose makes the poison”.

To complement pharmacological methods, a second approach to reveal the functions of individual receptors has been the development of null mice, through targeted gene disruption or deletion. Multiple null or “knockout” (KO) mice have been created targeting ERα [45, 46], ERβ [46–48] and most recently GPER [49–52]. As no overt or extreme phenotypes in GPER KO mice similar to those of ERα KO mice were initially observed, questions arose as to the functions of GPER as an estrogen receptor [53]. However, many recent studies have revealed significant phenotypic alterations in GPER KO mice in both normal and pathophysiology. Importantly, these studies not only reveal the effects of GPER deficiency, they also demonstrate that numerous estrogen-mediated effects are absent in GPER KO mice, underscoring the role of GPER as a mediator of estrogen action. In this review, we will provide an overview of the recent discoveries regarding the roles of GPER in estrogen-related physiology and disease based on the use of GPER KO mice, highlighting the physiological, pathological and therapeutic implications of GPER expression and activity.

Estrogen Receptor Knockout Mice

Several exhaustive reviews have described the phenotypes of mice lacking ERα, ERβ, and ERα/β double knockout mice [54–57]. The phenotypes exhibited in ERα KO mice affect multiple organ systems in adult mice, although embryonic organogenesis is normal [58]. Loss of ERα leads to complete infertility in both males and females, attributed to a combination of altered mating behavior and physiological deficiencies in the gonads of both sexes that prevent ovulation and spermatogenesis [58–62]. The female reproductive tract is characterized by a hypoplastic uterus and a lack of responsiveness (proliferation, imbibition) to estrogen [63]. Similarly, the mammary glands in ERα KO mice develop normally during the fetal period, but no further ductal elongation occurs at puberty [64]. Other phenotypes include impaired long bone growth [65–67], loss of vascular/cardiac protection to injury [68], increase in white adipose tissue (adipocyte hypertrophy and hyperplasia) and associated glucose intolerance and insulin resistance in males and females [69].

ERβ KO mice display few overt phenotypes compared to ERα knockouts, and gene expression analysis demonstrates that each receptor regulates expression of distinct genes
suggesting that the two classical estrogen receptors have little redundancy. ERβ KO females are subfertile due to reduced ovulation [47], an ovarian phenotype distinct from that of ERα KO females. Other more subtle phenotypes that do not interfere with normal physiology include impaired terminal differentiation of mammary epithelia during late pregnancy [71] and prostate hyperplasia in older males [72, 73]. Both of these phenotypes are associated with increased Ki-67 expression, suggesting that ERβ functions to restrict proliferation and promote differentiation.

A second set of ERα and ERβ knockout mice were created [46] in light of the fact that a truncated but transcriptionally competent ERα transcript is detectable at low levels in the ERα KO mice [74]. These mice phenocopy the original ERα and ERβ KO mice, as well as the ERα/β double KO mice, in most respects [46]; however, functional redundancy was observed in ERα/β double KO ovaries, along with a transformation of granulosa cells into a cell type resembling Sertoli cells in the testis. Remarkably, alternatively spliced transcripts have been detected in both the original [47] and the second [46] ERβ KO mice. To resolve the function of ERβ, a third KO was created and confirmed to be a true null [48]. Female mice display infertility due to ovulation defects, while males are infertile due to unknown causes [48].

Despite the widespread phenotypes associated with loss of ERα, a small subset of estrogenic responses have been reported in ERα KO or ERα/β double KO mice, raising the intriguing possibility that another estrogen receptor such as GPER may mediate these activities. Some of these responses can be attributed to the residual truncated or alternative spliced transcripts present in the original ER KO mice [75]; in other instances classical ER involvement has been convincingly ruled out. For example using mRNA differential display, the expression of several genes was increased or decreased by estrogen treatment in ERα KO uteri [76, 77]. The gene products include proteins involved in calcium homeostasis, chaperone proteins, and proteins that regulate the Wnt signaling pathway, although a subsequent microarray analysis of a global deletion of ERα exon 3 revealed minimal residual estrogenic responses in the uterus [78]. In a model of cardioprotection during ischemia/reperfusion, wild type mice or mice lacking ERα or ERβ responded to estrogen treatment with improved recovery and reduced infarct size, but estrogen treatment did not lead to cardioprotection in GPER KO mice [79]. Moreover, estrogen attenuated early-stage atherosclerosis in an ERα-independent manner [80], although ERα is involved in the protective effects of estrogen in late-stage disease [68]. ERβ involvement was not assessed; however, other studies have found no evidence for ERβ function in atherosclerosis protection [81]. Finally, estrogen protects pancreatic β-cells from apoptosis, but in ERα/β double KO mice, estrogen continues to afford partial protection, whereas GPER KO mice demonstrate increased sensitivity to apoptotic stress, with the GPER-selective agonist G-1 affording protection in wild type mice similar to that of estrogen [82]. Collectively, these results reinforce the notion that while estrogen exerts a plethora of physiological effects through the classical estrogen receptors, specific estrogen-dependent activities are independent of ERα and ERβ.
GPER Knockout Mice

The identification of a G protein-coupled estrogen receptor, together with the realization that some estrogen-dependent physiological outcomes occur independently of classical ERs, prompted several groups to create genetically modified models of GPER through targeted gene deletion. A total of four such mice have been reported, each using a slightly different approach. Three of these used a strategy that deleted the entire GPER coding region, which is contained within one exon. All three knockouts are confirmed to be true nulls, while the fourth, which also encodes a lacZ reporter, retains expression of the C-terminal portion of the protein. For simplicity, in this review, we will refer to the GPER knockout mouse models as GPER KO1 [52], GPER KO2 [50], GPER KO3 [49], and GPER-LacZ [51].

GPER KO1 was created by disrupting the GPER gene using traditional positive (Neomycin resistance)/negative (thymidine kinase sensitivity) selection methods [52]. The full GPER gene, including coding and non-coding regions, was excised during targeted deletion, leaving the Neo cassette behind. Correct targeting was confirmed with restriction digest mapping and Southern blotting and sequencing of cloning junctions. GPER KO2 was created using a targeting vector containing the complete GPER open reading frame flanked by loxP sites, a loxP-flanked neo cassette for positive selection, and an upstream diphtheria toxin (PDK-DTA) negative selection cassette [50]. Once correct targeting was confirmed by Southern blotting and PCR, GPER and neo were excised by Cre recombinase. GPER KO3 was created using a strategy similar to that used to create GPER KO2, in that the complete GPER open reading frame (flanked by loxP sites) and neo selection cassette (flanked by frt sites) were completely removed following confirmation of homologous recombination [49]. Another difference between the three complete knockouts is that homologous recombination was performed in embryonic stem (ES) cells derived from the C57Bl/6N strain to create GPER KO3, while ES cells derived from the 129 strain were used for GPER KO1 (129SvEvTac) and GPER KO2 (129X1/SvJ x 129S1), followed typically but not always by a minimum of 5–6 (or better 10) generations of backcrossing to the C57Bl/6 strain.

The fourth GPER knockout line, GPER-lacZ, was created through homologous recombination that replaced the first 349 bp of the GPER open reading frame (amino acids 20-136) with an insertion cassette that includes the full-length lacZ transcript and the neo resistance cassette [51]. Like GPER KO1 and GPER KO2, homologous recombination was accomplished in 129 ES cells, and once germline transmission was confirmed, mice were backcrossed 6 generations onto the C57Bl/6 strain. In addition to lacZ expression, real-time PCR reveals the presence of a neoR:5′-terminal truncated GPER fusion transcript. Therefore the C-terminal ~65% of the GPER protein, including transmembrane domains 3–7, is expressed in this knockout model. It is not clear if this truncated protein would be non-functional, partially functional, or functionally dominant-negative.

Initial analyses of the four GPER KO mouse models revealed no overt phenotypes in viability or reproductive function. Nevertheless, as will be described in detail herein, more detailed histopathological analysis and response to various physiologic challenges has revealed several phenotypes in metabolism and disease progression. At first glance it may appear that the different KO models display distinct phenotypes; however, in most cases...
different protocols and analyses have been performed, and in only a few instances have similar or identical studies been conducted on multiple knockouts by different labs.

Reproductive Functions

As estrogen was discovered in studies aimed at identifying sex hormones, it is of no surprise that the role of estrogen in the female reproductive system is one of its best-characterized functions. As described above, ERα is responsible for the majority of estrogen’s effects on the female and male reproductive tracts, including maintenance of the hypothalamic-pituitary-gonadal axis, uterine estrogenicity (epithelial proliferation, water imbibition, and induction of estrogen-responsive genes), ductal elongation in the mammary gland, and spermatogenesis. ERβ mediates some reproductive functions, mainly to orchestrate ovulation and male fertility through an unknown mechanism. In contrast, all of the GPER KO mouse models exhibit normal fertility and reproduction, suggesting that GPER does not contribute to normal reproductive physiology [49–52], although GPER mRNA expression was detected in the uterus, ovary, and mammary gland of wild type mice [49]. More detailed inspection of the GPER KO3 mouse model revealed no differences in uterine or mammary gland histopathology. GPER KO3 uteri responded to ovariectomy and estrogen challenge as well as wild type uteri as measured by water imbibition, epithelial cell height and proliferation, and expression of estrogen-responsive genes that are reported to be ERα-independent [76, 77]. Similarly, GPER KO3 and wild type mammary gland response to hormonal stimulation (estrogen or estrogen + progesterone following ovariectomy) in terms of morphometry, proliferation, and estrogen-dependent gene expression was equivalent [49].

Three studies have examined the role of GPER in uterine function using pharmacologic perturbation with GPER-selective compounds. In one study no difference was seen in proliferation using the GPER-selective agonist G-1 in wild type mice [83]; however, quantitation of the proliferation results was not described. In a second study, estrogen and G-1 significantly increased proliferation 18 hours after administration, although the magnitude of the increase compared to sham-treated mice was distinct for the two agonists (~17-fold for estrogen vs. ~3-fold for G-1). Importantly, the GPER antagonist G15 blocked estrogen-dependent proliferation by about 50% in a dose-dependent manner [39], suggesting that GPER contributes to estrogen-dependent proliferation. G-1 treatment did not increase in water imbibition in either study. The differing observations of the two studies may be due to the length of agonist treatment. It has been reported that peak uterine epithelial proliferation occurs approximately 18 hours following estrogen treatment (Newbold et al, 2001), and therefore this time point was used by Dennis et al. [39], as opposed to 3 treatments, 24 hours apart [49, 83]. Indeed, measured estrogen-induced proliferation reached 60% of total epithelial nuclei 18 hours after a single estrogen injection [39], but only ~16% after 3 estrogen injections over 3 days [49], potentially resulting in an undetectable effect of G-1. Finally, a third study found evidence for GPER-ERα crosstalk in uterine responses. Very high concentrations of G-1 (1,000-fold greater than the concentration used to detect an increase in proliferation) displayed an inhibitory effect on estrogen-induced proliferation and imbibition, in part due to phosphorylation of ERα (serine 118; [84]). Collectively, these studies demonstrate that estrogen-dependent uterine epithelial proliferation is in part GPER-
dependent, while many other estrogen-dependent uterine responses attributed to ERα were intact in GPER KO mice.

**Immune functions**

Estrogens, as well as therapeutic anti-estrogens, regulate the development, maturation and function of the immune system [85–87]. GPER expression has been documented in various immune cells, including B and T cells, monocytes/macrophages and neutrophils, suggesting that certain actions of estrogens in the immune system could be mediated by GPER [40, 52, 88, 89]. The first study to describe the creation of (GPER KO) mice in 2008 used these mice to determine whether GPER played a role in estrogen-induced thymic atrophy, a condition that can occur during pregnancy or prolonged estrogen exposure and is characterized by reduced thymus weight (atrophy) and cellularity, as well as alterations in CD4 and CD8 T cell populations [90]. Previous studies with ERα KO mice had indicated that ERα only partially mediated the effects of estrogen on the thymus [91], whereas studies with ERβ KO mice revealed little effect of this receptor [92]. Interestingly, although GPER KO1 mice displayed no gross immunological defects, a role for GPER in estrogen-mediated thymic atrophy was identified [52]. In a direct comparison of ERα KO to GPER KO1 mice, estrogen-induced thymic atrophy was attenuated not only in ERα KO mice as previously observed, but also in GPER KO1 mice [52]. More detailed analyses revealed that estrogen-induced thymic atrophy involved both a developmental block as well as apoptosis. In fact, whereas ERα exclusively mediated the early developmental blockage of thymocyte development (specifically CD4/CD8 double-negative thymocytes), GPER was required for apoptosis of double-positive thymocytes. This result was further supported through the use of the GPER-selective agonist G-1, which induced thymic atrophy through thymocyte apoptosis without the developmental blockage ascribed to ERα. Although another report has observed an increased apoptosis rate of naïve T cells in GPER-LacZ reporter mice not challenged with estrogen [51], a lack of global effects of G-1 [83] and GPER expression [93] on the thymus has also been reported. The reasons for these apparently differing results remain unclear.

Estrogens are also receiving increased attention as anti-inflammatory therapeutic agents, particularly in multiple sclerosis, where disease symptoms are attenuated during pregnancy, suggesting a protective effect of estrogen(s) [94]. Consequently, a clinical trial of estriol for multiple sclerosis therapy is ongoing (ClinicalTrials.gov, [95]). Employing the murine experimental autoimmune encephalomyelitis (EAE) model, in which estrogens exhibit both protective (i.e. preventive) [96] and therapeutic [97] effects, estrogen-mediated protection, as assessed by clinical score, was substantially decreased in GPER KO1 mice, with a complete absence of estrogen-mediated protection from white matter damage in GPER KO1 mice (Fig. 1A) [98]. A role for GPER was further supported employing G-1 treatment with the EAE model, which resulted in protection against both the clinical and histological manifestations of EAE, similar to the effects observed with estrogen [40, 98]. Although the effects of G-1 were completely absent in GPER KO1 mice, demonstrating a requirement for GPER and the selectivity of G-1 for GPER, the protective effects of estrogen itself were only partially absent, suggesting that estrogen acts through mechanisms involving both ERα and GPER. Importantly however, activation of GPER alone (with G-1) in wild type mice
completely reproduced the effects of estrogen, suggesting that ERα activation is not essential when stimulating only GPER [98]. The effects of G-1 have not been examined in ERα KO mice, which would reveal whether G-1-mediated protection requires ERα expression and perhaps basal activity. Multiple mechanisms of GPER-mediated protection have been observed including enhanced suppressive activity of regulatory T cells through upregulation of programmed death 1 [98] as well as inhibition of macrophage inflammatory cytokine production [40]. In addition to the protective effects of estrogen itself, the therapeutic effect of ethinyl estradiol in established EAE was absent in GPER KO1 mice but remained in ERα KO mice, and was associated with increased IL-10 production [99]. Finally, the estrogen-dependent protective effects of vitamin D3 in EAE, were also absent in GPER KO1 mice and correlated with decreased anti-inflammatory cytokines, again suggesting GPER mediates the estrogen-dependent effects in this model [100]. Consistent with the anti-inflammatory role of GPER activation in EAE, aged GPER KO1 mice exhibit a pro-inflammatory state [101], possibly secondary to other metabolic dysfunctions (see section below on metabolic functions). Furthermore, G-1 stimulates de novo IL-10 production in pro-inflammatory Th17-polarized cells in vitro as well as following G-1 administration in vivo [102], and elicits Foxp3 expression (a marker of regulatory T cells) under Th17-polarizing conditions [103], all of which support an anti-inflammatory role of GPER activation.

**Cardiovascular and Renal Functions**

Estrogens play important roles in cardiovascular and renal function in both health and disease. The lack of estrogen resulting from menopause or oophorectomy is associated with an increased incidence of hypertension and cardiovascular disease compared to premenopausal women [104–106]. Premenopausal women also exhibit increased cardiovascular health compared to age-matched men, further indicating a protective role for estrogen. However, although the beneficial effects (cardiovascular, in particular) of estrogen came under question as a result of the Women’s Health Initiative (WHI) Trial of hormone replacement using conjugated equine estrogens (combined with medroxyprogesterone acetate, a synthetic progestin analog) [107, 108], more recent analyses, particularly for recently menopausal women, largely confirm the safety and efficacy of estrogen replacement therapy [109, 110].

The first reports to describe a cardiovascular role for GPER employing GPER KO mice were published in 2009 by Barton and colleagues [111] and Leeb-Lundberg and colleagues [50]. In the former study, the vasodilatory effect of the selective GPER agonist G-1, which was similar to that of estrogen, was absent in carotid arteries of GPER KO1 mice. Furthermore, the inhibitory effects of G-1 on serotonin-mediated contractions were absent in GPER KO1 mice. Consistent with the observed vasodilatory effects on isolated rings, acute intravenous injection of G-1 into normotensive Sprague-Dawley rats induced rapid reductions in mean arterial pressure (MAP). In the latter study, GPER KO2 mice displayed increased MAP at 9 but not 6 months of age, due in large part to a decrease in the WT mice at 9 vs. 6 months of age. Interestingly, acetylcholine relaxed K+-preconstricted aortic rings similarly in WT and GPER KO2 mice, unlike later results employing physiologically preconstricted rings (see below in this section). Differences in MAP were ascribed to a lack
of increase in lumen circumference in mesenteric resistance vessels of older GPER KO2 mice, suggesting a deficit in vessel diameter growth in GPER KO2 mice with age. Reports have also described that male, but not female, GPR30 KO3 or GPER-LacZ mice exhibit either impaired left ventricular cardiac function with enlarged left ventricles displaying both impaired contractility and relaxation [112] or decreased ejection fraction and fractional shortening, specifically in aging (male) mice [113].

Detailed studies of vascular function and reactivity in GPER KO1 mice were first described by Meyer et al. in 2012, comparing the effects of chronic global GPER deficiency to acute vascular effects with the GPER antagonist G15, with respect to both endothelium-dependent and -independent reactivity [114, 115]. In the aorta, GPER deficiency was associated with increased endothelial prostanoid-mediated vasoconstriction with no effect on endothelial nitric oxide (NO) activity. Direct thromboxane prostanoid (TP) receptor-mediated contraction was also increased in GPER KO1 mice. Acute GPER inhibition with G15 enhanced endothelium-dependent contractions and reduced NO activity (both basal and acetylcholine-induced) but had no effect on direct TP receptor activation [114]. In pressurized carotid arteries, GPER KO1 show enhanced constriction to endothelin, despite a decrease in vascular smooth muscle cell calcium mobilization, suggesting that GPER activation mediates a reduced myofilament force sensitivity to calcium [115]. In another study, the vasoconstrictor activity of peri-vascular adipose, a cyclooxygenase-derived thromboxane, was first identified in obese GPER KO mice [116]. In addition to studies of GPER KO mice, many additional studies have employed the selective GPER agonist G-1 to underscore the vasodilatory actions of GPER [117–121]. Finally, supporting a role for GPER in the regulation of vascular tone, a common (22%) hypofunctional genetic variant of GPER has been associated with increased blood pressure in women [122].

In epidemiological studies and animal models of cardiovascular diseases such as atherosclerosis and ischemia/reperfusion injury resulting from myocardial infarction and stroke, estrogen plays a highly protective role [123]. Interestingly, the anti-atherogenic effects of estrogen, particularly in the early stages of atherosclerosis, are independent of ERα [80]. Recent results demonstrate that GPER KO1 mice exhibit increased early atherosclerosis progression when fed a high-fat atherogenic diet, under both ovary-intact and ovariectomized conditions [124]. This corresponded with increased vascular macrophage (Fig. 1B) and T cell infiltration and decreased basal NO activity. Importantly, treatment with G-1 reduced plaque formation and macrophage accumulation, consistent with a protective role for GPER. The cardioprotective effects of estrogen following ischemia/reperfusion have also been shown to be dependent on GPER expression, with GPER KO2 mice, but not ERα KO or ERβ KO mice, lacking estrogen-mediated protection [79, 125]. Similarly, G-1 mediates cardioprotective effects through mechanisms involving ERK-mediated inhibition of mitochondrial transition pore opening [126]. Akt activation [127] and reductions in inflammatory cytokines [128]. Activation of GPER also improves diastolic function and left ventricular remodeling in hypertensive rats [129] and ameliorates cardiac function in isoproterenol-induced heart failure [130].

GPER activity is also involved in the renoprotective effects of estrogen. In the kidney, GPER is expressed predominantly in distal convoluted tubules and the Loop of Henle, with
lower expression in proximal convoluted tubules and little expression in collecting ducts, with varying expression levels and patterns throughout the estrus cycle [131]. Estrogen and G-1 in addition to the SERD ICI182,780 (Fulvestrant), an ER antagonist, stimulated rapid calcium signaling and H^+\text{ATPase} activity in renal tubules and isolated intercalating cells, with the effects of all three ligands absent in tubules and cells isolated from GPER KO2 mice (Fig. 1C) [132]. In a model of salt-induced hypertension with commensurate renal damage, G-1 reduced renal hypertrophy, improved creatinine clearance and reduced proteinuria, all in the absence of blood pressure effects, presumably due to the presence of endogenous estrogen [133, 134]. G-1 reduced tubular oxidative stress and induced megalin expression leading to improved protein reabsorption [134]. The acute renoprotective effect of estrogen following cardiac arrest and cardiopulmonary resuscitation, which involves neither ER\(\alpha\) nor ER\(\beta\) [135], was recently shown also not to involve GPER, based on the use of GPER KO1 mice and G-1 [136], suggesting that the renoprotective functions of GPER may be context-specific.

Metabolic Functions: Obesity and Diabetes

Among the first phenotypes reported for GPER KO mice was that they were obese (Fig. 1D) [111], glucose intolerant (Fig. 1E) [50] and exhibited increased sensitivity to diabetes-inducing treatments [82]. Estrogen is an important mitigant of these and other metabolic disorders in both humans and animal models [137] and thus it is not unexpected that GPER plays a role in multiple aspects of metabolic regulation.

In 2009, Barton and colleagues reported the observation that, in addition to the cardiovascular effects described above, both male and female GPER KO1 mice were obese, exhibiting excess abdominal adipose [111]. Interestingly, at about the same time, Leeb-Lundberg and colleagues reported that female (but not male) GPER KO2 mice exhibited a ~10% decrease in body weight at 19 weeks of age, with a similar decrease in overall body size [50], whereas Otto and colleagues observed no differences in body weight at 5 months of age in GPER KO3 mice [49]. Finally, Noppinger and colleagues reported that the overall body weight as well as lean and fat mass was unaltered in GPER-LacZ fusion mice [51], although female GPER-LacZ mice fed a high-fat diet exhibited lower HDL levels and excess fat accumulation in the liver [113]. Some of these apparent discrepancies may be due to the age of the mice studied, targeting strategy, genetic background, diet or possibly environmental factors. For example, a more in depth study by Sharma et al. of aging male GPER KO1 mice revealed that whereas the body weight difference was only about 7% in 6 month-old mice, this increased to almost 20% in 12 month-old mice, with unaltered body size (tibia length) [101]. A similar but slightly reduced weight difference was maintained through 24 months of age. In these mice both visceral and subcutaneous adipose increased by ~30–40% [101], with a three-fold increase in the peri-aortic adipose [116]. Dyslipidemia in male GPER KO1 mice, as evidenced by increased serum levels of total cholesterol, LDL cholesterol and triglycerides, occurred as the mice aged (24 vs. 12 months) [101]; whereas in young female GPER KO1 mice, an atherogenic diet resulted in increased total and LDL cholesterol levels [124]. Consistent with these observations in mice, epidemiological evidence for the regulation of LDL cholesterol through GPER activity in humans derives from carriers of a hypofunctional GPER polymorphism that correlates with higher

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circulating levels of plasma LDL cholesterol, potentially through the regulation of LDL receptor expression in the liver [138].

Similar levels of obesity in GPER-deficient mice have also been observed by Clegg and colleagues [139]. Importantly, the estrogen-mediated reductions in body weight and adipocyte size (which increase following ovariectomy) were greatly attenuated in GPER KO1 mice, consistent with a role for GPER in mediating certain metabolic actions of estrogen [139]. Decreased energy expenditure (measured as oxygen consumption) was also reported for GPER KO1 mice, along with decreased UCP1 and β3-adrenergic receptor expression in brown adipose [139], although no difference in food intake or locomotion was observed [101, 139], suggesting specific roles in peripheral energy metabolism. Nevertheless, central effects of GPER in the hypothalamus may also contribute to effects on overall obesity [139, 140] (see below). Additionally, the GPER-selective agonist G-1 inhibits adipogenesis [141] and decreases fatty acid synthesis and triglyceride accumulation in human and rodent islets and β-cells, suggesting roles in the development of adipose tissue and lipid metabolism in the pancreas [142].

In addition to lipid metabolism and homeostasis, extensive data indicate that GPER also plays important roles in estrogen-mediated glucose homeostasis [11], particularly with respect to pancreas/islet/β-cell function and survival. Estrogen exhibits numerous important effects in the pancreas, including stimulating insulin synthesis [143, 144] and secretion [145, 146], enhancing β-cell survival [11, 147] and islet survival following transplantation [148], and reducing lipotoxicity [142, 149]. In 2009, Mauvais-Jarvis and colleagues employed a streptozotocin-induced model of type 1 diabetes with ERα, ERβ and GPER KO mice to demonstrate that both ERα and GPER mediated pancreatic islet survival [82]. Specifically, female GPER KO1 mice exhibited a loss in protection from streptozotocin-induced diabetes by endogenous estrogens and the GPER-selective agonist G-1 protected from ROS-induced apoptosis as effectively as estrogen in both murine and human islets, with the protective effect of G-1 absent in islets isolated from GPER KO1 mice [82]. Furthermore, G-1, as well as ERα- and ERβ-selective estrogenic ligands, increase survival of human islets following xenotransplantation into mice, but only the ER-selective ligands improved islet revascularization [148].

In 2009, Leeb-Lundberg and colleagues reported that whereas ovary-intact female GPER KO2 mice were glucose intolerant at 6 months of age, male mice at the same age were unaffected [50]. However, Sharma et al. subsequently revealed that male GPER KO1 mice are insulin resistant at 6 months of age and only become glucose intolerant as they age [101], possibly explaining the loss of observed glucose tolerance phenotypes in other studies of GPER KO mice [51, 82]. In female GPER KO2 mice, impaired glucose tolerance was associated with decreased insulin expression and glucose-stimulated secretion in pancreatic islets [50]. Furthermore, following ovariectomy, estrogen replacement failed to preserve serum insulin levels [50] in GPER KO 2 mice and glucose tolerance [139] in GPER KO1 mice as in wild type mice. In order to determine the effects of GPER specifically on pancreatic insulin release, both isolated islets [50, 150] and β-cells [150] have been examined. Whereas estrogen stimulated insulin and inhibited glucagon secretion in islets isolated from wild type male and female mice, these effects were absent in islets from GPER
KO2 mice (Fig. 1F) [50, 150]. Furthermore, the GPER-selective agonist G-1, similar to estrogen, stimulated insulin secretion from islets isolated from wild type but not GPER KO1 mice; in addition, the GPER-selective antagonist G15 inhibited insulin secretion by both estrogen and G-1 [150]. Taken together, these results demonstrate extensive roles for GPER in metabolic regulation, and specifically in many of the effects mediated acutely or chronically by estrogen.

Cancer

Estrogen promotes the proliferation of breast epithelial cells at puberty, leading to epithelial ductal outgrowth, as well as tumor cell proliferation in breast cancer. Extensive evidence supports a role for ERα in these activities [151, 152], and indeed standard medical practice includes determination of ERα expression following a breast cancer diagnosis, in order to evaluate suitability of anti-estrogen therapy (e.g., SERMs) [153]. However, the greater benefits observed from aromatase inhibitor (AI) therapy [154], which prevents estrogen production, compared to tamoxifen therapy, which inhibits estrogen activating classical ERs, suggest the possibility that estrogen promotes breast cancer progression through additional mechanisms.

The question of whether GPER might contribute to breast cancer progression was explored in several ways. In vitro studies have demonstrated that GPER activation stimulates MAPK, PI3K/Akt and related survival/proliferation signaling pathways, concomitantly inducing cell growth, in multiple GPER-expressing breast cancer cell lines [24, 25, 155–157]. Examination of GPER expression in human breast tumors has revealed a correlation between GPER expression level with both tumor size and metastasis [158]. Furthermore, an inverse correlation was observed between GPER expression and disease-free survival in patients initially presenting with GPER-positive tumors, and treated only with tamoxifen [159]. Collectively these results support the notion that GPER may contribute to breast cancer progression, and importantly given that tamoxifen is a GPER agonist, may contribute to some forms of tamoxifen resistance.

To date, only one study has investigated GPER function in breast cancer progression in a physiologically intact model: GPER KO1 mice (backcrossed onto the FVB strain for 10 generations) interbred with mice transgenic for the polyoma middle T antigen (PyMT), expressed under the control of the mouse mammary tumor virus (MMTV) long terminal repeat [160]. MMTV-PyMT transgenic (Tg) mice express the PyMT oncoprotein in the mammary gland, and develop mammary tumors with high fidelity [161]. The MMTV-PyMT Tg model of breast cancer progression mimics human breast cancer in terms of histopathology, temporal gene expression changes during tumor progression, loss of/ decrease in ERα expression [162, 163] and estrogen-responsiveness [163, 164], multistep tumor progression, metastasis to the lung, and gene expression patterns [165, 166]. GPER deficiency in this model did not affect tumor latency or hyperplasia in early stages of tumor onset [160]. However, GPER KO1 had smaller tumors at later stages of tumorigenesis, with reduced proliferation and histologic features of less aggressive tumors. Importantly, GPER KO1 mice displayed decreased metastasis to the lung (Fig. 1G) [160]. This represents the first study to provide direct evidence for a functional role of GPER in breast cancer.

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progression. Collectively these results, combined with in vitro experiments and retrospective human breast cancer analysis, suggest that GPER expression positively influences breast tumor progression in certain settings, and also suggest that GPER should be investigated as a therapeutic target.

GPER has also been linked to the progression of other cancers, including endometrial and ovarian cancer, where estrogen exposure increases risk [167, 168]. GPER expression strongly correlated with poor prognosis in endometrial and ovarian cancer in women [169, 170]. The Hec50 cell line expresses GPER but not ERα or ERβ, and in a xenograft model of endometrial tumorigenesis, Hec50 tumor growth increased with G-1 and estrogen treatment, while estrogen-mediated growth was inhibited by the GPER antagonist G36 [36]. Studies of endometrial or ovarian cancer utilizing GPER KO models have not been reported; however, they may one day illuminate the role of GPER in these hormone-responsive cancers as with the breast cancer model.

**Neurological Functions**

Estrogens, both systemic and synthesized locally in nervous tissue [171], mediate effects throughout the central and peripheral nervous system. Functions include regulation of the hypothalamus-pituitary-gonadal axis, sexual/reproductive behavior, synaptic plasticity, mood, memory, cognition, pain sensation, energy metabolism, temperature regulation and neuroprotection from injury [172]. GPER expression has been documented in multiple regions of the central and peripheral nervous system of both female and male rodents, including the cortex, hippocampus, hypothalamus, nuclei of the midbrain, the trigeminal nuclei and cerebellum Purkinje layer of the hindbrain, the anterior, intermediate and neural lobes of the pituitary, as well as the spinal cord and dorsal root ganglia [173–175]. Kelly and colleagues have described a putative membrane-associated estrogen receptor that is coupled to desensitization of GABA_B receptors in hypothalmic proopiomelanocortin neurons. Having previously demonstrated the effect was maintained in ERα KO, ERβ KO and ER double KO mice [176], in 2008, they also demonstrated that GPER KO were not defective in this estrogen-mediated effect, suggesting the involvement of another as-of-yet unidentified estrogen receptor [177].

Clegg and colleagues, in examining the metabolic effects of GPER deficiency, observed that the weight gain observed in female GPER KO mice (discussed above) was associated with a decreased anorectic response to leptin [139]. Importantly, plasma insulin and leptin levels, as well as estrogen and testosterone levels, and hypothalmic leptin receptor levels were unaltered although there was a ~25% decrease in hypothalmic ERα mRNA expression. Furthermore, the prandial effect of CCK, which is sexually dimorphic and greater in females, and results in anorexia, was reduced in female but not male GPER KO mice (Fig. 1H). In addition, whereas food intake varies across the female estrus cycle, GPER KO mice displayed no such variations, suggesting GPER plays a role in rapid estrogenic enhancement of satiety. A potential mechanism for this resulted from the demonstration that the estrogen-induced phosphorylation of ERK1/2 in the basal medial hypothalamus is absent in ovariectomized female GPER KO mice [139].
Roles for GPER in anxiety and stress responses have also been demonstrated employing GPER KO mice. Kastenberger and Schwarzer have recently described highly sexually dimorphic effects of GPER deficiency [178]. In particular, male GPER KO3 mice displayed increased exploratory drive in multiple tests, whereas female GPER KO3 mice displayed estrous-stage dependent anxiolytic effects in the open field test with a shift in the cycle-dependent fluctuations of basal cortisone levels and stress coping responses. Thus, the effects of GPER deficiency on behavior are clearly complex and require further investigation.

In support of studies with GPER KO mice, a plethora of studies have investigated the behavioral functions of GPER using GPER-selective agonists and antagonists. For example, Kwon et al. recently demonstrated that estrogen stimulated STAT3 phosphorylation in GPER-expressing, but not in ERα- or ERβ-expressing cells, and that estrogen- (and G-1)-stimulated phosphorylation in hypothalamic neurons was blocked by the GPER antagonist G15 [140]. Furthermore, cerebroventricular injection of G-1 increased STAT3 phosphorylation in the arcuate nucleus, and was associated with a decrease in food intake and body weight gain [140]. G-1 also induces depolarization of spinal cord neurons [174], stimulates mechanical hyperalgesia, [179] and mediates visceral hypersensitivity [180]. In vivo studies further demonstrated that G-1 replicates the effects of estrogen in enhancing neuronal survival following global or local ischemia in the brain [181, 182], improves cerebral microvascular function following hypoxia and reperfusion [183] and improves immunosuppression following stroke [184]. G-1 also attenuates serotonin receptor signaling in the paraventricular nucleus of the hypothalamus and reduces responses to oxytocin and adrenocorticotropic hormone, consistent with a role for GPER in mood disorders [185]. In addition, G-1, like estrogen, exhibited antidepressant properties in a mouse model of depression, with effects by both ligands inhibited by the GPER-selective antagonist G15 [39]. Although G-1 did not alter the estrogen-mediated inhibition of negative feedback by luteinizing hormone, and furthermore knockdown of GPER in the mediobasal hypothalamus did not alter lordosis behavior in rats [186], a recent report did in fact demonstrate that G-1 promotes lordosis in mice [187]. Together, these results suggest that GPER plays extensive roles in neurological function and that GPER agonists could represent a new therapeutic approach for stroke, behavioral, as well as chronic neurodegenerative diseases [188].

**Skeletal Functions**

Estrogen plays multiple roles in the growth and metabolism of bone [189]. This is exemplified by the loss of bone mass and density (osteoporosis) in women following menopause, and the effectiveness of hormone replacement therapies, as well as SERMs, to prevent or limit osteoporosis [190]. In men as well as mouse models, deficiency in ERα or aromatase, which converts testosterone into estrogen, is associated with low bone density and delayed growth plate closure [191]. GPER expression has been demonstrated in growth plate cartilage, declining as puberty progresses [192], and in osteoblasts, osteocytes and osteoblasts [193]. In preosteoblasts, GPER expression was upregulated by Runx2, a regulator of osteoblast differentiation, and GPER knockdown inhibited osteoblast proliferation [194]. Together, these results suggested a role for GPER in bone metabolism.
Surprisingly, few studies have examined the role(s) of GPER in skeletal metabolism and physiology. In 2009, Leeb-Lundberg and colleagues, in their original characterization of GPER KO2 mice, reported a small age-dependent decrease in femur length and overall size (crown-rump) in female but not male GPER KO2 mice [50]. Subsequently, the same group demonstrated that in ovariectomized female GPER KO2 mice, estrogen treatment failed to reduce longitudinal skeletal growth or decrease growth plate height as it did in wild type mice, although other parameters (bone mineral density and cortical bone thickness were unaffected in the GPER KO2 mice [93]. In 2011, Oz and colleagues reported that male GPER KO1 mice not only exhibited increased lean body mass and body fat, but greater femur length, bone mineral density, trabecular bone volume, cortical thickness and surface mineralization [195]. In addition, the growth plate of male GPER KO1 mice showed greater proliferation and under osteogenic conditions, bone marrow cells produced fewer alkaline phosphatase-positive colonies in early differentiating cultures, but increased mineralized nodule deposition in mature osteoblast cultures. No effects on osteoclast function were observed. Importantly, levels of IGF-1 in the serum were unaltered, suggesting that in male mice, GPER regulates multiple aspects of bone mass, size and microarchitecture [195]. Taken together, these results demonstrate sexually dimorphic effects of GPER in bone that require further study.

Conclusions

Our understanding of the roles of GPER in physiology and disease has expanded greatly in the last decade, and even more so in the last five years. In part, this has been a result of the development and more widespread use of GPER KO mice. Importantly, numerous functions of estrogen, though clearly not all, have been shown to be absent in GPER KO mice, in support of its designation as a physiologically relevant estrogen receptor (Table I). Furthermore, the effects of G-1 in animal models are absent in GPER KO mice, revealing the specificity of G-1 for GPER in vivo. Nevertheless, studies using knockout mice have inherent limitations. For example, effects in global KO mice are prone to complex interactions when a target is expressed in multiple interacting tissues. Compensatory changes during development may also obscure certain aspects of a protein’s inherent function(s). Genetic differences in strain background can impact phenotypes [196]. Furthermore, multiple approaches to the deletion of the Gper gene as well as environmental factors (chow, phytoestrogens, climate control, altitude, etc.) could result in mice with somewhat varying phenotypes in different labs. The development of inducible tissue-specific conditional KO mice will be an important addition to the arsenal of tools available to study GPER function, allowing tissue-specific deletion of GPER in adult mice, reducing compensatory developmental effects and the complexity of multiple interacting systems. The creation of double (ERα/GPER) or even triple (ERα/ERβ/GPER) KO mice, though challenging, could provide additional information regarding the possible overlapping functions of these receptor systems. To date, the use of pharmacological agents has provided an important complimentary approach to the study of GPER KO mice. In many cases, GPER-selective antagonists recapitulate the effects observed in GPER KO mice, whereas GPER-selective agonists demonstrate the opposite effect, often mimicking the effects of estrogen. Importantly, pharmacological agents can be used with greater ease in vivo, and in
species not amenable to genetic manipulation, though with their own caveats (stability, half-life, metabolism, selectivity, etc.). Nevertheless, when used in combination, these multiple approaches are slowly but surely unmasking the physiological and potential therapeutic roles of GPER.

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References


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## Highlights

- GPER KO mice reveal multiple roles for GPER in estrogen physiology.
- Pharmacological manipulation of GPER supports many of these functions.
- GPER-selective agonists and antagonists represent novel therapeutic agents.
- GPER targets include obesity, diabetes, cardiovascular diseases, stroke and cancer.
Figure 1.
GPER deficiency leads to multiple phenotypes and loss of estrogen responsiveness of multiple tissues. A. The estrogen-mediated decrease in white matter damage, a result of immune infiltration and demyelination, in a murine model of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE) is absent in GPER KO1 mice [98]. B. GPER KO1 mice fed an atherogenic diet show increased vascular infiltration of macrophages (CD68-positive cells) associated increased atherosclerosis [124]. C. Renal intercalated cells from GPER KO2 mice are deficient in estrogen (E2)-, ICI182,780 (ICI)- and G-1-mediated calcium mobilization. Calcium mobilization to progesterone (P4) was unaltered in GPER KO2 mice, similar in magnitude to estrogen-mediated calcium mobilization in WT cells [132]. D. GPER KO1 mice exhibit increased body size/obesity (left) and adiposity (right, as measured by MRI) [101]. E. Upon aging, male GPER KO1 become glucose intolerant [101]. F. Estrogen (E2)-mediated insulin secretion from islets is absent in both male and female GPER KO2 mice [50]. G, glucose. G. GPER KO1 mice exhibit reduced metastasis in a PyMT-MMTV model of mammary tumorigenesis [160]. H. Female GPER KO1 mice display a lack of cholecystokinin (CCK)-mediated satiety/appetite suppression (an estrogen-sensitized effect) as measured by food intake [139]. Reproduced (in some cases with minor modifications for clarity) with permission from the indicated sources.
**Table I**

Loss of Estrogen-mediated Effects in GPER KO mice

<table>
<thead>
<tr>
<th>System</th>
<th>Measurement</th>
<th>Observation in GPER KO*</th>
<th>GPER KO model and Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nervous</td>
<td>Prevention in EAE†</td>
<td>E2 ↓, G-1 (−)</td>
<td>KO1 [98]</td>
</tr>
<tr>
<td></td>
<td>Therapy in EAE</td>
<td>Ethinyl estradiol (−)</td>
<td>KO1 [99]</td>
</tr>
<tr>
<td></td>
<td>pERK activation in basal medial hypothalamus</td>
<td>E2 (−)</td>
<td>KO1 [139]</td>
</tr>
<tr>
<td></td>
<td>Cholecystokinin- and leptin-mediated satiety</td>
<td>(−) (females)</td>
<td>KO1 [139]</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Vascular dilation</td>
<td>E2 ↓, G-1 (−)</td>
<td>KO1 [111, 197]</td>
</tr>
<tr>
<td></td>
<td>Reduction in myocardial infarct size</td>
<td>E2 (−)</td>
<td>KO2 [79]</td>
</tr>
<tr>
<td></td>
<td>atherosclerosis</td>
<td>↑ (females)</td>
<td>KO1 [124]</td>
</tr>
<tr>
<td>Kidney</td>
<td>Calcium flux in renal intercalated cells</td>
<td>E2 (−), G-1 (−), ICI182,780 (−)</td>
<td>KO2 [132]</td>
</tr>
<tr>
<td>Pancreas</td>
<td>STZ-induced diabetes†</td>
<td>↑ (females)</td>
<td>KO1 [82]</td>
</tr>
<tr>
<td></td>
<td>Insulin secretion by islets and β-cells</td>
<td>E2 (−), G-1 (−)</td>
<td>KO1 &amp; KO2 [50, 150]</td>
</tr>
<tr>
<td></td>
<td>Decreased glucagon secretion</td>
<td>E2 (−)</td>
<td>KO2 [50]</td>
</tr>
<tr>
<td></td>
<td>Islet cell survival</td>
<td>G-1 (−), E2 effect compensated by ERα</td>
<td>KO1 [82]</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Glucose tolerance (ovx)</td>
<td>E2 (−)</td>
<td>KO1 [139]</td>
</tr>
<tr>
<td></td>
<td>Serum insulin (ovx)</td>
<td>E2 (−)</td>
<td>KO2 [50]</td>
</tr>
<tr>
<td></td>
<td>Body weight (ovx)</td>
<td>E2 ↓</td>
<td>KO1 [139]</td>
</tr>
<tr>
<td></td>
<td>Adipocyte size (ovx)</td>
<td>E2 ↓</td>
<td>KO1 [139]</td>
</tr>
<tr>
<td>Immune</td>
<td>Thymocyte apoptosis</td>
<td>E2 (−)</td>
<td>KO1 [52]</td>
</tr>
<tr>
<td>Skeletal</td>
<td>Longitudinal bone growth (ovx)</td>
<td>E2 (−)</td>
<td>KO2 [93]</td>
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<tr>
<td></td>
<td>Decreased growth plate height (ovx)</td>
<td>E2 (−)</td>
<td>KO2 [93]</td>
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<td></td>
<td>Bone mineral density, trabecular bone volume</td>
<td>↑ (males)</td>
<td>KO1 [195]</td>
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<tr>
<td>Cancer</td>
<td>Mammary tumor growth and metastasis</td>
<td>↓</td>
<td>KO1 [160]</td>
</tr>
</tbody>
</table>

* E2/G-1 (−), ↑, ↓, (-) effect of estrogen (E2) or G-1 is lost (−) or diminished (↓) in GPER KO mice; ↑, ↓, (-) (females/males): the indicated estrogen-responsive disease state or physiological effect is increased (↑), decreased (↓), or absent (−) in female/male GPER KO mice.

† ovx, ovariectomized. EAE, experimental autoimmune encephalomyelitis; STZ, streptozotocin.